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THE INITIAL CALCIFICATION PROCESS IN SHELL-REGENERATING *TEGULA* (ARCHAEOGASTROPODA)

CHARLENE REED-MILLER

Department of Geology, Florida State University, Tallahassee, FL 32306

Abstract

Shell regeneration was induced in the marine archaeogastropod, *Tegula,* by cutting a window in the first body whorl of the shell. At six hour intervals for six days after the shell window was cut, the mantle, foot, and hepatopancreas were prepared for transmission electron microscopy, and the shell window was prepared for scanning electron microscopy. Transmission electron microscopy of the three tissues showed an increase in rough endoplasmic reticulum, Golgi complexes, and mitochondria, followed by the appearance of three types of inclusions. Later, intracellular space increased and spherites were visible. Scanning electron microscopy showed initial crystal deposition in the shell window to be in the form of small doubly-pointed crystallites associated with an organic membrane. These spindle-shaped crystals were frequently aggregated into radiating clusters or rosettes which coalesed until a thin sheet of mineralized material covered the shell window, within six days of shell injury.

Introduction

The regeneration or repair of molluscan shell is a subject of great interest. Most of the studies of repair of mineralized tissues in molluscs have concerned terrestrial or freshwater species (Wagge, 1951; Tsujii, 1960; 1976; Beedham, 1965; Saleuddin, 1967; Abolins-Krogis, 1968; Saleuddin and Wilbur, 1969; Kapur and Gupta, 1970; Meenakshi *et al.,* 1975; Blackwelder and Watabe, 1977). Reports on shell regeneration in marine molluscs include work on the cephalopod, *Nautilus macromphalus* (Meenakshi *et al.,* 1974), and the bivalve, *Mytilus edulis* (Meenakshi *et al.,* 1973; Uozumi and Ohata, 1977; Uozumi and Suzuki, 1979). One impression from these studies is that marine molluscs require more time to repair their shells than do terrestrial or freshwater species.

Meenakshi *et al.* (1974) report that it takes 45 days for shell regeneration to occur in *Nautilus,* and 30–32 days must elapse following shell injury before the first evidence of mineral deposition occurs in *Mytilus* (Meenakshi *et al.,* 1973). Furthermore, it takes at least eight weeks before the regenerated shell takes on a normal appearance in *Mytilus* (Meenakshi *et al.,* 1973; Uozumi and Ohata, 1977; Uozumi and Suzuki, 1979). These chronologies are impressively long compared to the time required for substantial calcium deposition in the land snails *Helix* and *Otala, e.g.,* two to three days (Wilbur, 1973). The freshwater snail, *Heliosoma,* and the freshwater bivalve, *Anodonta,* require a somewhat longer time for mineral deposition—at least five days (Chan and Saleuddin, 1974) and about 14 days (Tsujii, 1976) respectively. But these freshwater molluscs still repair damaged shell faster than their marine counterparts.

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Preliminary observations showed that initial mineral deposition occurred about 24 to 48 hours after the creation of a shell window in the first body whorl of the marine snail, *Tegula* (Reed-Miller *et al.,* 1980). On the average, six days were required for a thin sheet of mineralized tissue to cover the shell window (Reed-Miller, unpub. ob.). This paper describes the events of the first six days of shell repair in the marine archaeogastropod, *Tegula.* Both scanning and transmission electron microscopy were used. Preliminary accounts of this work were presented to the American Society of Zoologists (Reed-Miller, 1981).

Materials and Methods

Snails, *Tegula funebralis* and *Tegula eiseni,* were obtained from the Pacific Biomarine Laboratories, Inc., Venice, CA. They were maintained in aquaria in filtered, aerated sea water from the Gulf of Mexico (32 ppt), at 15°C. The animals were fed marine algae from a laboratory culture.

A 4 mm^2 section of shell was carefully removed from the first body whorl of the shell using a Dremel "Moto-tool," jeweler's saw, and a triangular file. Care was taken not to injure the underlying tissue. The opening in the shell, or window, was covered with a small piece of a plastic coverslip, and that in turn was covered with warm dental wax, sealing the window from the external environment.

Small pieces of the mantle from directly underneath the shell window, foot, and hepatopancreas were carefully dissected from the animals six hours to six days after the window was cut. As controls, the same tissues from normal, non-regenerating snails were always prepared with those from shell regenerating animals. The experiments were repeated at least three times with at least four experimental animals examined each time.

Transmission electron microscopy

The soft tissues were dissected out and fixed at room temperature in 1% glutaraldehyde in filtered sea water (pH 7.2). The tissue was then washed three times in a 1:1 sea water:glass distilled water solution. Following the third wash, the material was postfixed for one hour in 1% osmium tetroxide in filtered sea water, rinsed with glass distilled water, dehydrated through a graded series of ethanol, taken through two changes of propylene oxide, and embedded in Medcast (Ted Pella, Inc., Tustin, CA). Silver to gold sections were cut with a diamond knife, and stained with uranyl acetate and lead citrate. The specimens were observed in a Philips 201 transmission electron microscope operated at 60 kV.

Scanning electron microscopy

After removal of the soft parts, the shell was preserved in 70% ethanol. Then the shell was carefully cut around the shell window with a rotary rock saw, until a small frame of shell (about 3 mm wide) surrounded the window on all sides. This frame and the shell window with the regenerated material were rinsed with distilled water and air dried. The samples were mounted on aluminum scanning electron microscopy stubs with nail polish, coated with 100–200 Å of gold palladium (60:40), using a E5100 Polaron Sputter Coater. The material was observed with a Cambridge S4-10 scanning electron microscope operated at 20 kV.

Results

The structures of the three tissues from normal, nonregenerating snails were unexceptional, and, in fact, identical to descriptions from transmission electron microscope studies of those tissues appearing in the literature (Abolins-Krogis, 1961; 1963; Tsujii, 1976; Watabe *et al.*, 1976). The purpose of this paper is to describe the ultrastructural changes in the tissues during shell regeneration. The sequence of events was consistent for each snail in the experimental group. However, the time after the shell window was cut until each ultrastructural change was seen showed some individual variation. Consequently, the results are outlined in time frames following the creation of the shell window.

14–48 hours of regeneration

Three ultrastructural changes took place in the soft tissues during this stage of shell regeneration. First, the amount of rough endoplasmic reticulum increased, typically in the form of whorls or spirals (Fig. 1). Second, the number of Golgi complexes also increased (Fig. 2), and third, juxtaposed with the Golgi complexes, were open vesicles containing condensed or fibrous material (Fig. 3).

The shell window had small, doubly pointed or spindle-shaped crystals in and on an organic membrane (Fig. 4). This was the first appearance of mineralized material in the injured area of the shell. Often these crystals were aggregated into radiating clusters or small rosettes (Fig. 5).

48–72 hours of regeneration

The predominant feature in the soft tissues during this phase of regeneration were membrane-bound clusters of vesicles or vacuoles (Fig. 6). These inclusions took several forms, some were aggregates of very dense vesicles with some of the

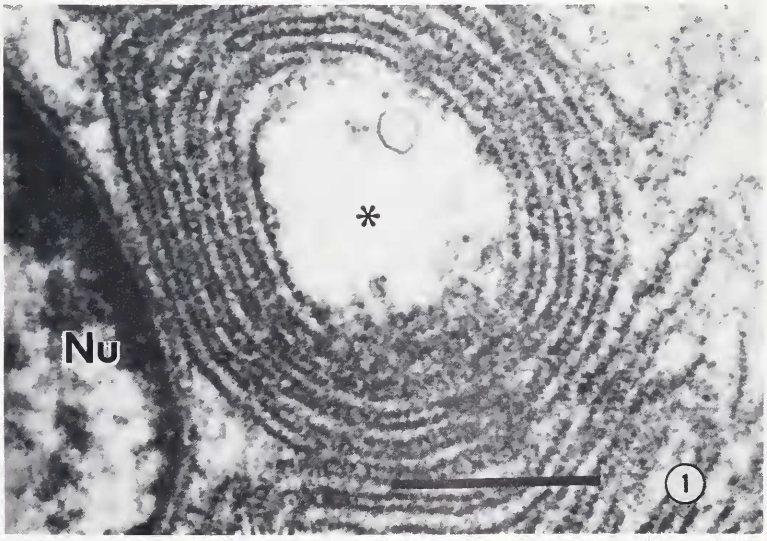

FIGURE 1. Foot epithelium, 36 hours of regeneration, showing a whorl of rough endoplasmic reticulum (*), Nu = nucleus, Bar = 500 nm.

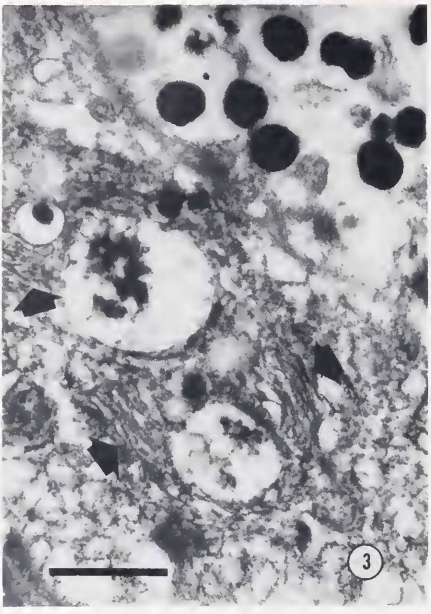

FIGURE 2. Mantle epithelium, 24 hours of regeneration, showing two Golgi complexes (arrows). Note the light fibrillar material near the Golgi complexes. Bar = 1 μm.

FIGURE 3. Foot epithelium, 48 hours of regeneration with several Golgi complexes (arrows) and associated vesicles containing some condensed material. Bar = 1 μm.

FIGURE 4. Scanning electron micrograph of the shell window, 48 hours of regeneration, showing small crystals associated with an organic matrix. Bar = 10 μm.

FIGURE 5. Higher magnification scanning electron micrograph of the shell window, 48 hours of regeneration, showing clusters of doubly-pointed crystallites. Bar = 10 μm.

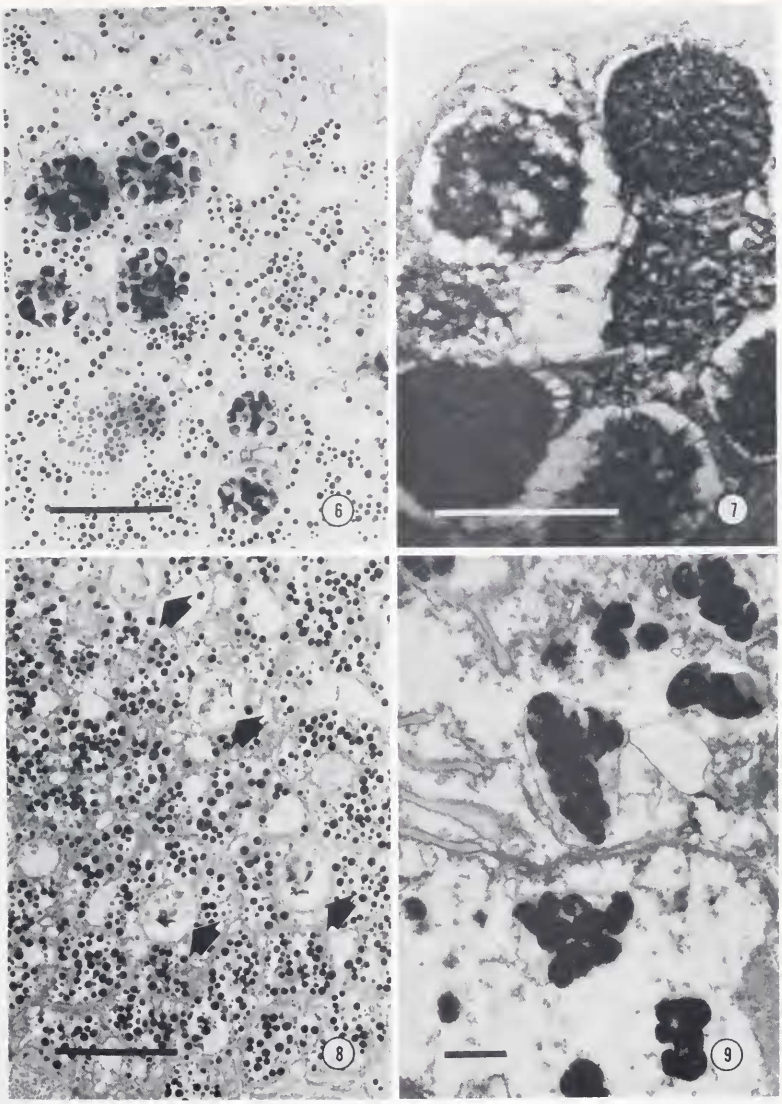

FIGURE 6. Mantle epithelium, 54 hours of regeneration, showing several inclusions containing dark vacuoles. The small dark droplets are melanin. Bar = 10 μm.

FIGURE 7. Mantle epithelium, 72 hours of regeneration, showing granular and fibrous material associated with a Type I inclusion. Bar = 500 nm.

FIGURE 8. Foot epithelium, 48 hours of regeneration, showing Type II inclusions (arrows). Small dark droplets are melanin. Bar = 10 μm.

FIGURE 9. Hepatopancreas, 72 hours of regeneration, with Type III inclusions. Bar = 1 μm.

individual vesicles appearing granular and connected to the other vesicles and the delimiting membrane by a fibrous network (Fig. 7, Type I inclusions). In another form (Type II inclusions), the entire inclusion was round, and the vacuoles were less electron-dense than Type I inclusions (Fig. 8). The third form consisted of aggregates of two to 15 or more dark vacuoles (Fig. 9, Type III inclusions). These

three inclusions were not corrolated with any particular tissue, that is, all three morphologies were found in all three of the tissues during this stage of regeneration.

The regenerated material in the shell window consisted of spindle-shaped crystals, and was virtually identical to the description for 14–48 hours of regeneration.

72 hours–six days of regeneration

As shown in Figure 10, transmission electron microscopy of the mantle, foot, and hepatopancreas showed widened intracellular spaces and spherules. A fibrous network linked the cores of the spherules with the surrounding membrane (Fig. 11).

By this stage of regeneration, *e.g.,* as early as 72 hours, but no later than six days after the shell window was cut, a thin sheet of material formed by the coalescence of spindle-shaped crystals covered the shell window (Figs. 12 and 13).

Discussion

The present study shows that the mantle, foot, and hepatopancreas of *Tegula* undergo ultrastructural alterations during the first six days of shell repair. Each of these tissues has been implicated in shell repair and calcification (Abolins-Krogis, 1970a, b; Burton, 1972; Watabe *et al.,* 1976; Tsujii, 1976; Watabe and Blackwelder, 1980). However, few studies concern the involvement of all three tissues at the same time.

Since of the three tissues studied, the mantle is the one usually associated with molluscan shell formation (Wilbur, 1964; 1972; 1976; Crenshaw, 1980), the ultra-

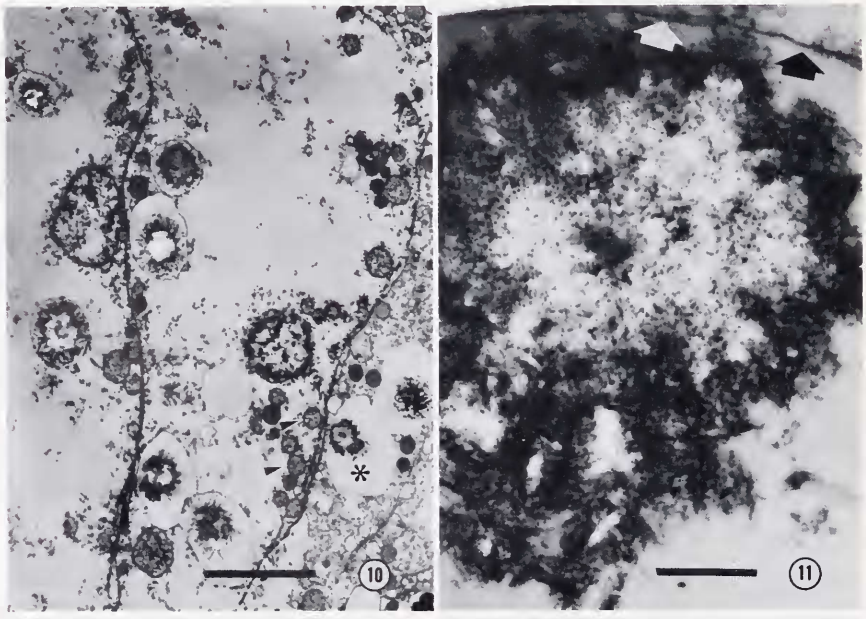

FIGURE 10. Mantle, 4 days of regeneration, showing wide intracellular spaces and spherules. Arrowheads indicate some mitochondria. Bar = 5 μm.

FIGURE 11. Mantle, 4 days of regeneration, showing at higher magnification the spherule indicated with a * in Figure 10. Note the membrane (arrow) and the granular-fibrillar appearance of the dark material surrounding the lucent core. Bar = 100 nm.

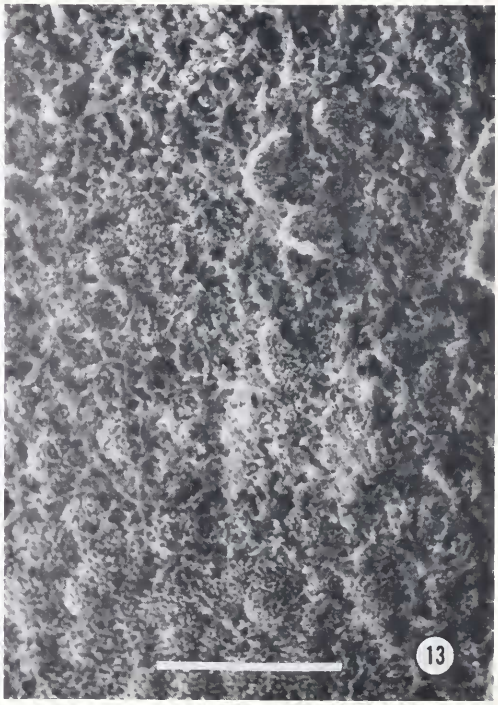

FIGURE 12. Scanning electron micrograph of the shell window, 6 days of regeneration, showing rosettes of spindle-shaped crystals associated with an organic matrix (arrowheads). Bar = 10 μm.

FIGURE 13. Scanning electron micrograph of the shell window, 6 days of regeneration, showing the coalescence of spindle-shaped crystals and rosettes to cover the window. Bar = 100 μm.

structural observations are discussed with regard to the role of this organ. The function of the foot and the hepatopancreas in shell repair will be compared to previous work of these tissues in other molluscs.

The mantle edge is the region actively involved in shell growth (Tsujii, 1976; Crenshaw, 1980). In this study, the site of shell regeneration was the first body whorl of the shell which lies over the central zone of the mantle—an area not usually involved in shell formation (Tsujii, 1976; Crenshaw, 1980). Following the initiation of shell regeneration, this zone of the mantle showed ultrastructural changes that may indicate an increased role in shell maintenance. These include a proliferation of rough endoplasmic reticulum, typically in whorls or spirals. Comparable changes have been reported in the mantle edge of *Heliosoma* (Saleuddin, 1976), *Helix* (Saleuddin, 1970, Fig. 11), and marine bivalves (Bubel, 1973a, b).

An increase in Golgi complexes was evident in the mantles of the shell-regenerating gastropods in this study. Moreover, as was the case for rough endoplasmic reticulum, increased numbers and activity in Golgi complexes have been described in active regions of the mantle in other molluscs. For instance, during periostracum or shell repair in *Mytilus edulis* and *Helix pomatia* (Saleuddin, 1970; Bubel, 1973c). Precursors of the periostracum were observed in Golgi cisternae in the gland cells of the mantle of *Littorina* (Bevelander and Nakahara, 1971), and Watabe *et al.* (1976) found that the formation of calcareous spherules in *Pomacea paludosa* was preceded by large vacuoles near the Golgi apparatus.

There was also an increase in mitochondria. Saleuddin (1970) noted 24 hours after shell injury to *Helix pomatia,* that the number of mitochondria increased. The calcium cells of *Pomatia paludosa* continued numerous mitochondria (Watabe *et al.,* 1976). In fact, these workers suggest that their finding lends support to the notion that the mitochondria are involved in the uptake and release of calcium and phosphate for calcification (Spiro and Greenspan, 1969; Lehninger, 1970; Saleuddin, 1970; Elder and Lehninger, 1973; Becker *et al.,* 1974). Certainly this could also be the role of the increased mitochondria in the tissues of shell-regenerating *Tegula.*

Three types of inclusions were noted. Their appearance was preceded by proliferation of rough endoplasmic reticulum and Golgi complexes. Vacuoles in the inclusions and fibrous, matrix-like material were found in close proximity to the Golgi complexes. Presumably, these inclusions are derived from the reticulum-Golgi complex system as in other calcifying systems (see Spangenberg, 1976; Watabe *et al.,* 1976; and Simkiss, 1980, for examples and discussions of the Golgi vesicle-reticulum system in calcification).

Distinctions as to the possible functions of each of the three types of inclusions are impossible to make with the current data, but some speculation on their roles is possible. It is conceivable that one role may be to provide matrix material for the deposition of mineral for regenerated shell. The most likely candidates for this part are the Type I and Type II inclusions, based on the similarity in their ultrastructural appearance to inclusions that do serve as mineral deposition sites in other systems (See Watabe *et al.,* 1976, and Watabe and Blackwelder, 1980, for discussions of Golgi-derived vacuoles and vesicles in another gastropod under normal and shell-regenerating conditions).

The Type III inclusions may represent another morphology of calcifying vesicle or vacuole, or it may be involved in cellular detoxification. Calcification and mineralization involve a high degree of cellular activity, and there must be a way of ridding the cells of the resulting waste. Mason and Simkiss (1982), Kingsley and Watabe (1982), and Simkiss (1980) discuss similar inclusions in invertebrates and their role in detoxification.

If the inclusions are involved in providing sites for deposition of calcium in this system, the calcium must be mobilized to the site of shell regeneration. It is interesting that the appearance of the inclusions is followed by the occurrence of lucent cored spherules. These spherules may be similar to the naturally decalcified spherule found in the calcium cells of *Pomacea paludosa* (Watabe *et al.,* 1976; Watabe and Blackwelder, 1980).

Since the ultrastructural picture was similar to that for the mantle, it would be repetitive to consider in detail the changes in the foot and the hepatopancreas reported here. However, there are some points to be made about the possible roles of these tissues in shell repair. For example, when calcareous spherule development was not evident, prominent Golgi complexes and abundant rough endoplasmic reticulum and mitochondria occurred in the calcium cells of the foot and the albumin-capsule gland complex as well as in the mantle of *Pomacea paludosa* (Watabe *et al.,* 1976). Later work showed that spherule calcium was used for shell regeneration (Watabe and Blackwelder, 1980). The spherules described in the foot of the shell-regenerating *Tegula* in this study may be contributing calcium for shell repair as did those in *Pomacea.*

The role of the hepatopancreas in molluscan shell regeneration has been debated. Burton (1972) suggested that calcium from the hepatopancreas of *Helix* was used for shell repair, but did not show it to be mobilized. Work on the hepatopancreas of shell-regenerating *Helix pomatia* showed calcium spherites developed in protein-

containing Golgi saccules (Abolins-Krogis, 1970a), and alterations in the ultrastructure of this organ (Abolins-Krogis, 1970b; 1972). Simkiss (1980) found that spherules from the mantle and the foot of *Helix aspersa* were soluble in saline, while those from the hepatopancreas were not. He suggested different functions for the two types of spherules—those from the foot and the mantle would be involved in mineralization, while the more insoluble ones would be responsible for cellular detoxification (Simkiss, 1980; Mason and Simkiss, 1982). Campbell and Boyan (1976) suggested that the function of calcium spherules in the gastropod hepatopancreas is as a phosphate reserve. The hepatopancreas of *Tegula* may provide calcium for shell repair, as well as detoxifying the animal and providing phosphate.

Regenerated shell may be different from or similar to the normal shell ultrastructure (Saleuddin and Wilbur, 1969; Wilbur, 1972; Wong and Saleuddin, 1972). The small doubly-pointed crystals and the stellate shapes they formed have been described for both normal and regenerated shell in other molluscs. Spherulitic aggregates have been shown in another archaeogastropod, *Cittarium pica* (Wise and Hay, 1968a, b; Erben, 1971), and in the regenerated shell of *Pomacea paludosa* and *Cepaea nemoralis* (Blackwelder and Watabe, 1977; Watabe, 1981). The crystals formed during early shell regeneration in *Nautilis macromphalus* are doubly-pointed, associated with an organic matrix, and form stellate aggregates which grow until a spherulitic prismatic layer is formed (Meenakshi *et al.*, 1974). Therefore, the spindle-shaped crystals described in the regenerated shell of *Tegula* appear to be a common morphology of calcium carbonate in some archaeogastropods as well as in regenerated molluscan shell.

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